

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

Lasse LEINO et al.

Serial Number: 10/534,988

Group Art Unit: 1614

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Examiner: Simmons, Chris E.

For: PHARMACEUTICAL COMPOSITION FOR INTRACELLULAR
ACIDIFICATION WITH CIS-UROCANIC ACID

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Jarmo LAIHIA, declare as follows:

1. I am one of the inventors of the invention described and claimed in U.S. Patent Application S.N. 10/534,988 ("the application") and am aware the claims of the application have been rejected as anticipated by U.S. Patent No. 5,494,676 to Stab et al. ("Stab et al.").

2. I am the director of research and development for BioCis Pharma Oy, the assignee of the application. I, or those working under my supervision, performed experiments to measure the pH of compositions disclosed in Stab et al.

A. Two cream formulations were obtained from Trekos Cosmetics Ltd., Söderkulla, Finland. On information and belief, Table 1 sets forth the composition of the cream formulations, which is in accordance with col. 11, lines 30-45 of Stab et al.:

Table 1. Composition of Cream Formulations

No.	Name	Ingredient	Percent by Weight
1	Cream A	Ceteareth-20	3.00
		Cetylstearyl alcohol	8.00
		Vaseline, GP 9	10.00
		Mineral oil	10.00
		Urocanic Acid ¹	1.00
		Demineralized Water	68.00
2	Cream B	Ceteareth-20	3.00
		Cetylstearyl alcohol	8.00
		Vaseline, GP	10.00
		Mineral oil	10.00
		Demineralized Water	69.00

B. The pH of the creams was measured using a Biotrode electrode connected to a Knick Calimatic pH-Meter 766.

¹The urocanic acid ("UCA") was a 50/50 mixture of *cis*-UCA and *trans*-UCA, in accordance with Col. 7, lines 25-29 of Stab et al. The *cis*-UCA was 99.95% pure and was produced by BioCis Pharma Oy, Turku, Finland. The *trans*-UCA was 99.75% pure, and was obtained from Acros Organics BVBA, Geel, Belgium.

The pH electrode was calibrated 1-2 days prior to measurement using two standard reference solutions having a pH of 4.00 and 7.00, respectively.

C. The cream formulations were brought to ambient room temperature (22-23 °C). A 200 mg sample of each cream formulation was diluted in 400 µl of ultrapure water. The pH of the diluted sample was recorded when the reading remained stable (within ±0.03 pH units for at least 20 seconds) under continuous slow stirring. The results are listed in Table 2 below:


Table 2. pH of Cream Formulations

No.	Name	pH
1	Cream A	4.74
2	Cream B	7.79

3. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true. These statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful

false statements may jeopardize the validity of the application
or any patent resulting therefrom.

Signed this 30th day of January, 2008.

Signed: 

Name: Jarmo Laihia

The Inhibition of EGF-dependent Proliferation of Keratinocytes by Tyrphostin Tyrosine Kinase Blockers

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Abstract. Protein tyrosine kinase blockers of the tyrphostin family inhibited the EGF-dependent proliferation of human and guinea pig keratinocytes grown in culture and induced their growth arrest. These blockers also significantly inhibited the growth of epidermal keratinocytes, but not of dermal cells, in whole skin organ culture from both guinea pig and human origin. The antiproliferative activity of these tyrphostins correlated quantitatively with their potency as inhibitors of EGF receptor autophosphorylation and the EGF-dependent protein phosphorylation of intracellular tar-

get proteins in the keratinocyte. Furthermore, no significant cell cytotoxicity or reduction in serine and threonine phosphorylation of many intracellular polypeptides were observed upon incubation of the cells with tyrphostins like AG213. The complete growth arrest induced by the tyrphostins is fully reversible and upon their removal the keratinocytes resumed their growth with the original growth rate. Because of the nontoxic nature of these compounds and their growth-arresting properties, we suggest their use as agents to treat hyperproliferative conditions of human skin.

PHOSPHORYLATION of proteins on tyrosine residues is a key biochemical reaction that mediates a large variety of cellular signals (14, 32), including the control of the cell cycle and cell differentiation. Many of the cellular plasma membrane receptors, such as the receptors for EGF, PDGF, and insulin, possess an integral, intracellular, tyrosine kinase moiety that is activated upon the binding of its specific ligand to the extracellular domain of the receptor (28, 32). The involvement of abnormally high protein tyrosine kinase (PTK)¹ activity with a large variety of growth-related disease states has already been established (5). In many cases, the enhanced PTK activity of oncogene products or the overexpression of their normal counterparts was found to be essential for their transforming activity (5, 24, 28). Hyperproliferation of cells leading to nonmalignant growth is also often associated with enhanced PTK activity, for example, the enhanced PTK activity of PDGF receptor which results from its exposure to sustained levels of PDGF seen in atherosclerosis and restenosis (24). The overexpression of PTK oncoproteins, which results in enhanced kinase activity, can also alter the developmental pattern of cell types into which they have been introduced (3, 10, 29).

The involvement of PTKs in a wide range of disease states has thus led to the general concept that development of PTK blockers is a reasonable approach to combat hyperproliferative conditions which result from enhanced activity of PTKs

(17, 31). "Tailor-made" PTK inhibitors can be used as antiproliferative or anticancer agents (17, 31), where each family of blockers may be useful for a particular set of PTKs. We have recently shown that it is indeed feasible to synthesize a series of PTK inhibitors (Tyrphostins) that can discriminate by a factor of over three orders of magnitude between EGF receptor tyrosine kinase and insulin receptor tyrosine kinase (11, 31) and with intermediate potency against p210^{bc^r-abl} kinase (Anafi, M., A. Gazit, C. Gilon, Y. Ben-Neriah, and A. Levitzki, manuscript submitted for publication). These EGF receptor tyrosine-kinase-directed inhibitors block EGF-dependent Ca²⁺ signaling and phosphorylation of phospholipase C γ (20, 21) as well as EGF dependent proliferation of transformed cells (18, 31). Furthermore, these studies revealed a quantitative correlation between the tyrosine kinase inhibitory potency in vitro and the antiproliferative activity of these tyrphostins on cells (18, 31).

Among the processes governed by tyrosine kinases is, apparently, the normal development of human epidermal keratinocytes. These cells, which are capable of developing an almost normal differentiation program in culture (13), have been found to be dependent in proliferation on a variety of growth-promoting agents, in which EGF has a significant role (13, 19, 23, 30). The closely related TGF α , which is known to bind and activate the EGF receptor (8, 26), was also shown to be a potent mitogen of keratinocytes in culture (2) and to be actively secreted by keratinocytes in culture, and thereby might regulate their normal proliferation in an autocrine fashion (6, 7). Accumulating evidence indicates that there is an overexpression of the TGF α gene in psoriatic keratinocytes (9, 27) and accumulation of TGF α itself in

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1. *Abbreviation used in this paper:* PTK, protein tyrosine kinase.

psoriatic skin (27). This finding suggests the persistent autocrine activation of the EGF receptor which may play a major role in cell hyperproliferation in human psoriatic epidermis (9, 15).

In view of the role of EGF receptor in the normal human keratinocyte proliferation, and in its suggested possible involvement in abnormal proliferation typical of psoriasis, we were encouraged to examine whether the EGF receptor-directed Tyrphostins can be used to specifically inhibit the EGF receptor-dependent proliferation of human and guinea pig keratinocytes growing in cell and organ cultures.

Materials and Methods

Cell Cultures and Growth Inhibition by Tyrphostins

Tyrphostins. Tyrphostins were synthesized as described by us earlier (11, 12). Secondary- or tertiary-passaged human keratinocytes were seeded (5,000–10,000 cells/cm²) and grown on NIH 3T3 mitomycin-inactivated fibroblasts by the method of Rheinwald and Green (22, 23) (1.2×10^4 cells per 35-mm plate), in 2 ml DME/F12 (2:1) supplemented with 5% FCS (Beit-HaEmek, Israel) insulin, cholera toxin, hydrocortisone, transferrin, and triiodothyronine (13, 22). 24 h after seeding, media were replaced by new media or containing above tyrphostins at the indicated concentrations and 10 ng/ml EGF (mouse submaxillary glands, United States Biochemical Corporation, Cleveland, Ohio). Controls with media containing tyrphostins but without EGF were run in parallel. Before cell counting the cultures were treated with 0.01% trypsin in PBS for 5–10 min at 37°C to dislodge the feeder layer fibroblasts. Cells were counted in the Coulter Counter at days 4, 6, and 8 after seeding by treatment of the cultures with trypsin/EDTA/glucose solution (0.1%, 5 and 10 mM, respectively in PBS) to suspend the cells. 12 h before cell counting, [³H]thymidine (2 μ Ci/ml, specific activity 80 Ci/mmol, Amersham International, Amersham, UK) was added to a portion of the cultures and DNA synthesis was monitored as described (18). Data on cell numbers are shown in parallel at day 5. Each experimental point represents the average of triplicate 35-mm plates (Nunc, Denmark). Occasionally, human keratinocytes were grown in the above medium omitting the feeder layer for comparison. Guinea pig keratinocytes were isolated from young guinea pig ears. The ears were shaved, and their upper and lower halves were separated by forceps. The cartilage was removed, and the skin pieces (~ 5 mm²) were incubated 45 min at 37°C in PBS with 0.1% trypsin (2 \times crystallized). The epidermal cells were obtained by gentle scraping of the epidermis from the dermis, in growth medium containing 5% newborn calf serum. Guinea pig-derived cells were seeded at high density (0.5×10^6 cells/cm²) and grown on DME/F12 (2:1 vol/vol) with 10% FCS and 10 nM EGF without a 3T3 feeder layer or any other additions. Cell proliferation was estimated as in the case of human keratinocyte cultures omitting the pretreatment to dislodge feeder layer cells.

Inhibition of Cell Growth in Organ Cultures

Whole normal human foreskin pieces (~ 6 –7 mm²) were trimmed from excess dermal tissue and were incubated at 37°C, dermal side down, in 0.5 ml culture medium (DME/F12 as in cell culture) containing 0.25% FCS, tyrphostins (50–200 μ M), and EGF (10 ng/ml) as indicated. 24–72 h later, [³H]thymidine (10 μ Ci/ml) was added for another 24 h. At the end of the incubation period, the skin pieces were washed three times with PBS containing 10 mM thymidine (30 min each) and incubated overnight with 2.0 M NaCl solution containing 10 mM thymidine at 4°C. Epidermis was then separated easily from the dermis with a scalpel. After washing, drying on filter paper, and weighing the epidermal and dermal pieces they were counted for radioactivity in 4 ml scintillation liquid. Alternatively, guinea pig organ cultures were prepared from guinea pig ears by preparation of ear halves and removal of excess dermis and cartilage as above. Epidermal pieces varied in weight from 58 to 156 mg, while a larger variation (100–420 mg) was encountered in the dermis pieces. Also, variations were encountered in extent of epidermis and dermis labeling from skin to skin. The experiments were, therefore, conducted (see Fig. 6 C) so that each foreskin was divided into five small pieces which represent a whole experiment (control without EGF, control with EGF, and three pieces with EGF and tyrphostins at concentrations of 50, 100, and 200 μ M AG213). Results are expressed as [³H]thymidine incorporated per mg tissue.

Permeabilization and In Situ Phosphorylation of Cultured Keratinocytes

Normal human keratinocytes were grown in 35-mm culture dishes as described at higher seeds. 3–4 d after seeding (~ 70 –80% confluent), the remaining NIH 3T3 feeder cells were discarded by careful trypsinization, and the cultures were starved for 24 h in regular medium with 0.25% FCS. For the phosphorylation reaction, the wells were washed three times with PBS, and a reaction mixture containing 75 μ g/ml digitonin (crude; Sigma Chemical Co., St. Louis, MO), 15 μ M γ -[³²P]ATP (20,000 cpm/pmol; Amersham International), 1 mM PMSF, 10 μ g/ml leupeptin, 20 μ g/ml benzamide, 10 μ g/ml soybean-trypsin-inhibitor, 1.0 mM MgCl₂, 2.0 mM MnCl₂, 20 μ M ZnCl₂, 2 mM NaVO₃, with or without 10 ng/ml EGF, and tyrphostins, all in 50 mM Hepes, pH 7.5/saline buffer (600 μ l/35-mm dish), was added and incubated 5 min in a 37°C bath. The reaction was stopped on ice by washing with ice-cold PBS followed by 15 min incubation and swirling in 2 ml/dish cold stopping solution containing 1% Triton X-100, 50 mM Hepes saline, pH 7.5, 10 mM sodium pyrophosphate, 2.0 mM NaVO₃, 10 mM EDTA, 0.35 mM ATP, protease, and phosphatase inhibitors as above. Occasionally, the Triton X-100 extracts and remaining cytoskeletons (removed by rubber policeman from the plates) were analyzed for general phosphorylation without immunoprecipitation.

Immunoprecipitations

Cells in stopping solution after permeabilization were scraped off with a rubber policeman on ice. The lysate was centrifuged for 15 min in an Eppendorf centrifuge (14,000 rpm) at 4°C, and the supernatant (containing the triton-extracts and cytosols) was subjected to immunoprecipitation by incubating 2 h at 4°C (with continuous rotation 15 rev/min) with monoclonal antiphosphotyrosine antibodies coupled to agarose beads via protein A (Clone PT-66, a gift of Dr. Harry Langheheim, Sigma Immunochemicals Rehovoth, Israel). In several assays, the phosphorylation of the EGFR was followed by immunoprecipitation with anti-EGFR antibody (mAb 108 obtained from Rhone Poulenc Rorer Biotechnology, King of Prussia, PA). At the end of the incubation, the beads were washed thoroughly with washing buffer (same as the stopping solution, but without ATP and Triton X-100) and treated with SDS-PAGE denaturation buffer. The denatured reaction mixtures were then loaded on 5–15% gradient polyacrylamide gel and separated by electrophoresis (16). After drying of the gels, they were exposed to X-ray films (Curix RP-2, Agfa) in cassettes with intensifier screens. To quantitatively estimate the degree of phosphorylation of various protein bands, densitometry was performed using a Soft Laser densitometer Model SL-TRFF with Videophoresis II program (Biomed Instruments Inc., Fullerton, CA). The results were presented in arbitrary densitometer units (see Figs. 2 and 3) as a percentage of the densitometer units when maximal EGF stimulation is 100% value. Occasionally, the relevant bands were excised from the dried gels and counted in scintillation fluid to verify the densitometric analysis. Good correlation was found between the methods. Immunoprecipitation carried on in the presence of phenyl phosphate (10 mM) resulted in almost complete inhibition of the precipitation of phosphorylated polypeptides. It thus confirms that >95% of the phosphorylated bands do contain tyrosyl phosphate residues.

Results

Tyrphostins were tested for their growth inhibitory potential on normal human keratinocytes in culture grown on mitomycin-C-treated 3T3 fibroblasts as feeder layer (13, 22, 23). After 24 h growth in the absence of EGF, EGF and tyrphostins were added to the cultures and growth resumed for periods desired (see Materials and Methods). Tyrphostin-treated culture exhibited a strong reduction in cell proliferation rate as reflected by cell number, [³H]thymidine uptake, and colony size. Fig. 1 shows that the EGF-stimulated growth of keratinocytes is inhibited by three tyrphostins in the micromolar range, as determined by cell number. The IC₅₀ values are reproducible within $\pm 15\%$ between repetitive experiments. Similar inhibition profiles and IC₅₀ values were obtained when [³H]thymidine incorporation was measured

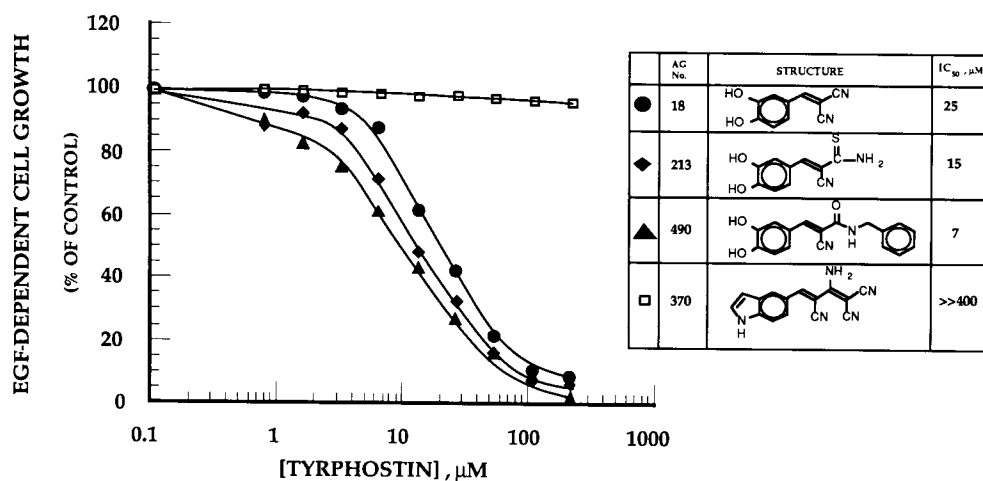


Figure 1. Inhibition of EGF-stimulated growth of human keratinocytes grown in culture by different tyrphostins. Normal human keratinocytes were grown as described in Materials and Methods. 24 h after seeding, the cultures were treated for another 72 h with and without EGF and with or without four different tyrphostins as depicted. Cell growth was monitored by counting with the Coulter counter. Number of cells in wells not containing EGF were subtracted from EGF-containing ones and the "EGF-dependent growth" was used to plot the tyrphostin inhibition.

100% EGF-dependent growth are values of cultures without added tyrphostins (1.1×10^5 cells/35-cm² plates averaged from three separate wells). Each point represents an average of a triplicate where the variation was <10%. IC₅₀ values varied some 15% between different experiments.

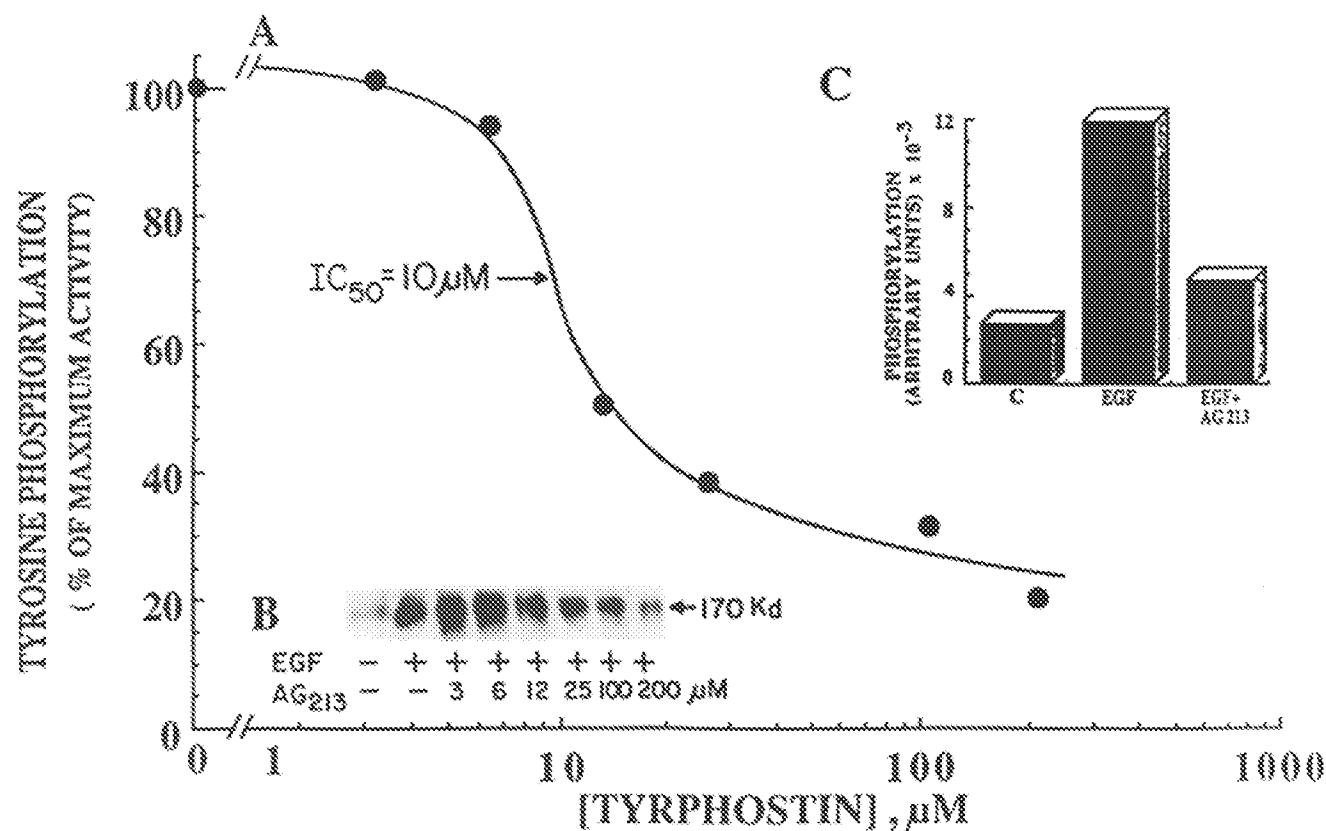


Figure 2. Inhibition of in situ autophosphorylation of EGF receptor by tyrphostin AG213 (RG 50864). In situ phosphorylation to cultured human keratinocytes was carried out as described in Materials and Methods. Lysates were subjected to immunoprecipitation using antiphosphotyrosine antibodies. Immunoprecipitates were dissolved in SDS-PAGE denaturation buffer, electrophoresed on polyacrylamide gels, and exposed to X-ray films as described in the experimental section. (A) Densitometry values obtained from autoradiogram. (B) Autoradiogram showing the 170-kD EGFR-phosphorylated band. (C) Maximal effect of AG213 (200 μM) compared to control (no EGF) or EGF. Quantitation was obtained by densitometry as explained in Materials and Methods and is presented in arbitrary densitometer units. Maximal EGF stimulation was 12,500 densitometer units which represent 1,209 dpm of ³²P labeling counted in the acrylamide slice, while control (no EGF) was 3,200 densitometric units which represent 288 dpm in the gel slice.

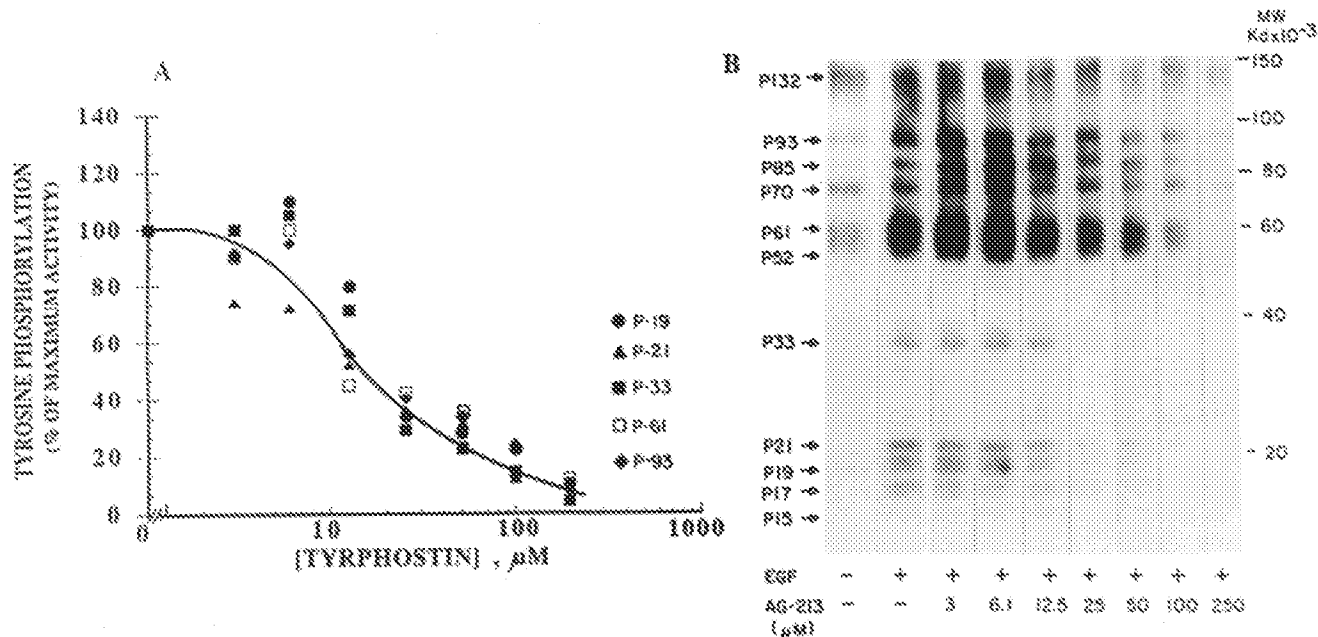


Figure 3. The inhibition of EGF-dependent phosphorylation of intracellular target proteins. In situ phosphorylation of normal human keratinocytes and immunoprecipitations was carried out as described in Fig. 2 and in Materials and Methods. Various polypeptides phosphorylated as induced by EGF and inhibited by AG213 (RG 50864) are shown after separation by SDS-PAGE. (A) The inhibition of EGF-dependent protein phosphorylation of individual protein bands by tyrphostin AG213 is depicted. The extent of phosphorylation was monitored by quantitative densitometry as described in the experimental section. (B) Autoradiogram showing the various phosphotyrosine containing intracellular substrates.

per cell. In an experiment similar to the one shown in Fig. 1, the EGF-dependent [^3H]thymidine incorporated was reduced from 10 ± 1 dpm/cell in the absence of tyrphostins to 0.2 ± 0.1 dpm/cell in the presence of $100 \mu\text{M}$ of the various tyrphostins shown in Fig. 1 at 4 d incubation. At the same time, the number of cells was found to be 4×10^4 cells in the inhibited cultures and 1.5×10^5 cells in uninhibited cultures (average of three plates). Thus, almost complete inhibition of the EGF-dependent cell proliferation occurred at high tyrphostin concentrations. Cell death, however, was minimal. After examining cells incubated for 48 h in the presence of EGF and then for another 4 d with 50, 100, and $150 \mu\text{M}$ tyrphostin AG 213 (RG 50864) and analyzed for cytotoxic effect (Trypan blue exclusion method), only $5 \pm 0.6\%$, $7.5 \pm 1.2\%$, and $12 \pm 3\%$ dead cells were found, respectively (average of three experiments). In uninhibited cultures only 5–7% dead cells were found after being grown for 8 d before trypsinization. Thus, tyrphostins are essentially cytostatic rather than cytotoxic (see also following sections). Tyrphostin AG370, which we have shown earlier (12) to be a poor inhibitor of EGFR kinase (12) ($\text{IC}_{50} \geq 820 \mu\text{M}$), is also ineffective in blocking EGF-dependent proliferation of keratinocytes (Fig. 1). To address the possible effect of the inactivated 3T3 fibroblasts present in the cultures as “feeder cells,” on the extent of inhibition we have grown human keratinocytes in the same medium, but without 3T3 cells added. At these heavy seeds, the cells could be grown in the absence of feeder cells (albeit at $\sim 50\%$ reduced rate) so we could test for the effect of tyrphostins. The same IC_{50} values for tyrphostin AG213 were found in the EGF-dependent proliferation in the absence of the feeder layer (data not shown). Similar inhibition pattern by tyrphostins was also found for guinea pig keratinocyte primary cultures grown in the absence of feeder layer (data not shown but see Fig. 5).

To further investigate the mechanisms of growth inhibition by tyrphostins, the effect of tyrphostins on the activity of the EGF receptor in these cells was examined. First, we tested the effect of these inhibitors on the phosphorylation of the receptor itself. Cells were serum starved to enhance EGF responsiveness, permeabilized, and challenged with $\gamma\text{-}[^{32}\text{P}]\text{-ATP}$, EGF, and tyrphostins. The EGF receptor was immunoprecipitated by antiphosphotyrosine antibodies and analyzed by SDS-PAGE. Fig. 2 shows the four- to fivefold increase in receptor phosphorylation by EGF and its inhibition by AG213 (RG 50864). This tyrphostin inhibits 80–90% of EGF activation, with an apparent IC_{50} of $10 \pm 2 \mu\text{M}$, similar to its IC_{50} value on cell growth inhibition (Fig. 1). Receptor phosphorylation in the absence of EGF (basal phosphorylation) is always detected and comprises 25–30% of maximal phosphorylation obtained in the presence of EGF. Similar results were obtained when anti-EGF antibody (mAb 108 obtained from Dr. J. Schlessinger [New York University Medical School and Rhone Poulenc Rorer, King of Prussia, PA] data not shown) was used. Also, since no phosphorylated EGF-R was detected when the immunoprecipitation with antiphosphotyrosine antibody was conducted in the presence of 10 mM phenyl phosphate (see Materials and Methods), we can conclude that tyrphostins inhibit the tyrosine phosphorylation of the receptor.

We next examined the effect of tyrphostin AG213 (RG50864) on the EGF-dependent phosphorylation of intracellular target proteins in the serum-starved and permeabilized keratinocytes. Fig. 3 depicts a typical experiment in which the EGF-activated phosphorylation of several of intracellular protein substrates and its inhibition by AG213 was examined. Most of the polypeptides showed a dramatic increase in phosphorylation (three- to sixfold) in response to EGF, although the extent of their “basal” phosphorylation varies. For example,

Table I. Potency of Tyrphostins as Antiproliferative Agents and EGF Receptor Kinase Blockers

Tyrphostin	Phosphorylation of exogenous or endogenous substrates		EGF-receptor autophosphorylation IC ₅₀		Cell proliferation IC ₅₀	
	K _i	IC ₅₀ , keratinocytes [‡]	Purified EGFR	Permeabilized keratinocytes	HER14	Keratinocytes
	μM	μM	μM	μM	μM	μM
AG 18	10 ± 1*	15	30 ± 3*	20 ± 2	15 ± 2 [†]	25 ± 3**
AG 213	0.8 ± 0.1*	10	8 ± 0.9*	10 ± 1	10 ± 1 [†]	15.0 ± 2**
AG 490	0.6 ± 0.2 [§]	7.0	0.2 [§]	—	3.5 [§] ± 0.5	7.0 ± 1.0**
AG 370	>500 [§]	>400	>500 [§]	—	—	—

* From ref. 11.

[‡] Obtained from experiments like the one shown in Fig. 3.

[§] From ref. 12.

^{||} Obtained in experiments similar to the one shown in Fig. 2.

[†] From ref. 18. HER 14 cells or NIH 3T3 cells into which EGF receptor has been stably transfected.

** Experiments performed as in Fig. 1.

p132, p70, and p61 had a rather high degree of constitutive phosphorylation in the absence of EGF. This is in contrast to polypeptides like p85, p33, and p19, as well as others, which show very low basal phosphorylation (5% to 15%) in the absence of EGF. The effect of AG213 was very pronounced, reducing the phosphorylation of most of these proteins with very similar apparent IC₅₀ values of 10.0 ± 3 μM (Fig. 3).

Table I summarizes the parameters of inhibition of the various tyrphostins used in different types of biochemical and cell growth assays in keratinocytes (this study) and in the HER 14 cells which overexpress EGF receptors and respond to the growth factor (18, 20). The inhibition of EGF receptor autophosphorylation, phosphorylation of protein substrates, and arrest in cell growth by tyrphostins show very similar IC₅₀ values. Moreover, the relative potency of the three tyrphostins in the biochemical (both in situ and in vitro) assays is identical to their relative potency in inhibiting EGF-dependent cell growth in two other EGF-responsive cell types (Table I). This correlation substantiates the role of EGF receptor in the growth of keratinocytes and that tyrphostin inhibition of keratinocyte's growth results from its inhibitory action on the tyrosine kinase activity of the EGF receptor.

To further examine whether tyrosine phosphorylation of cellular proteins is inhibited by the drugs used, we tested the effect of tyrosine AG213 on total phosphorylation (Fig. 4). The EGF-dependent phosphorylation profiles of the Triton X-100 extract and the cytoskeletons of the permeabilized cells were analyzed by SDS-PAGE and autoradiography (Fig. 4). Part of the extract was immunoprecipitated by antiphosphotyrosine and shown for comparison in parallel (see Fig. 4, A and B vs C, respectively). As can be seen, tyrosine phosphate-containing polypeptides phosphorylation represent a small fraction of the total protein phosphorylation (note days of exposure of autoradiograms in each fraction tested in Fig. 4, A–C, top). Tyrosine phosphate-containing polypeptides are barely detectable without prior immunoprecipitation with antiphosphotyrosine antibodies because of extensive phosphorylation (with the exception of P21 and sometimes P17 where EGF-dependent phosphorylation could be observed). Furthermore, tyrphostins did not affect the overall serine/threonine phosphorylation even at 100 μM , pointing again to the specificity and lack of general metabolic toxicity of these compounds. We have also analyzed the effect of AG213 and AG18 on protein kinase C in keratino-

cyte extracts in vitro. These tyrphostins inhibited PKC activity only at millimolar range of concentrations (data not shown). These findings also demonstrate the specific effect of tyrphostins on PTKs in these cells. Also, these compounds affect cAMP-dependent kinase only close to the mM concentration range (Posner, I., and A. Levitzki, unpublished observations).

We further investigated the effect of the tyrphostins on the morphology of keratinocyte cultures to gain insight on the mode of tyrphostins action. Arrest of keratinocyte growth by tyrphostins is much more pronounced on small, young cell colonies where terminal differentiation is still not apparent (Fig. 5, B and E). This growth arrest is reversible, since removal of the tyrphostin results in the resumption of keratinocyte proliferation within 24 h with the same growth rate (see rate of thymidine incorporation in the legend to Fig. 5). Upon removal of the inhibitors, the "basal" keratinocytes at the proliferative edge of the colony resume proliferation (Figure 5, C and F), leading to seemingly normal keratinization and differentiation. Thus, the cells remain viable in the presence of tyrphostins in agreement with the low cytotoxicity values given above. Furthermore, arrest of keratinocyte proliferation by tyrphostins appears not to affect the terminally differentiated cells in the colonies (Fig. 5, B and E).

To further examine the possibility that tyrphostins can be used as antiproliferative agents in whole skin, we examined their effect on epidermal cells in intact skin cultured in media (organ culture). Fig. 6 A shows a representative experiment showing that all the tyrphostins tested significantly inhibit most of the EGF-dependent (and some EGF-independent) proliferative ability of human epidermal cells in organ culture as measured by [³H]thymidine incorporation. This experiment represents the best EGF stimulation found in organ cultures. Fig. 6 B shows similar results for guinea pig skin. To be noted is the similarity in the order of potency of the tyrphostins in the different experimental systems (compare to Fig. 1 and Table I).

To determine more quantitatively the effect of the tyrphostins on the proliferation of epidermal and dermal cells in organ culture, we conducted nine independent experiments on human skin in organ culture using three concentrations of AG 213. Fig. 6 C shows averaged results of six experiments where significant EGF-dependent proliferation was found. Three conclusions can be drawn from the results. (a) Much higher concentrations of AG213 are needed in or-

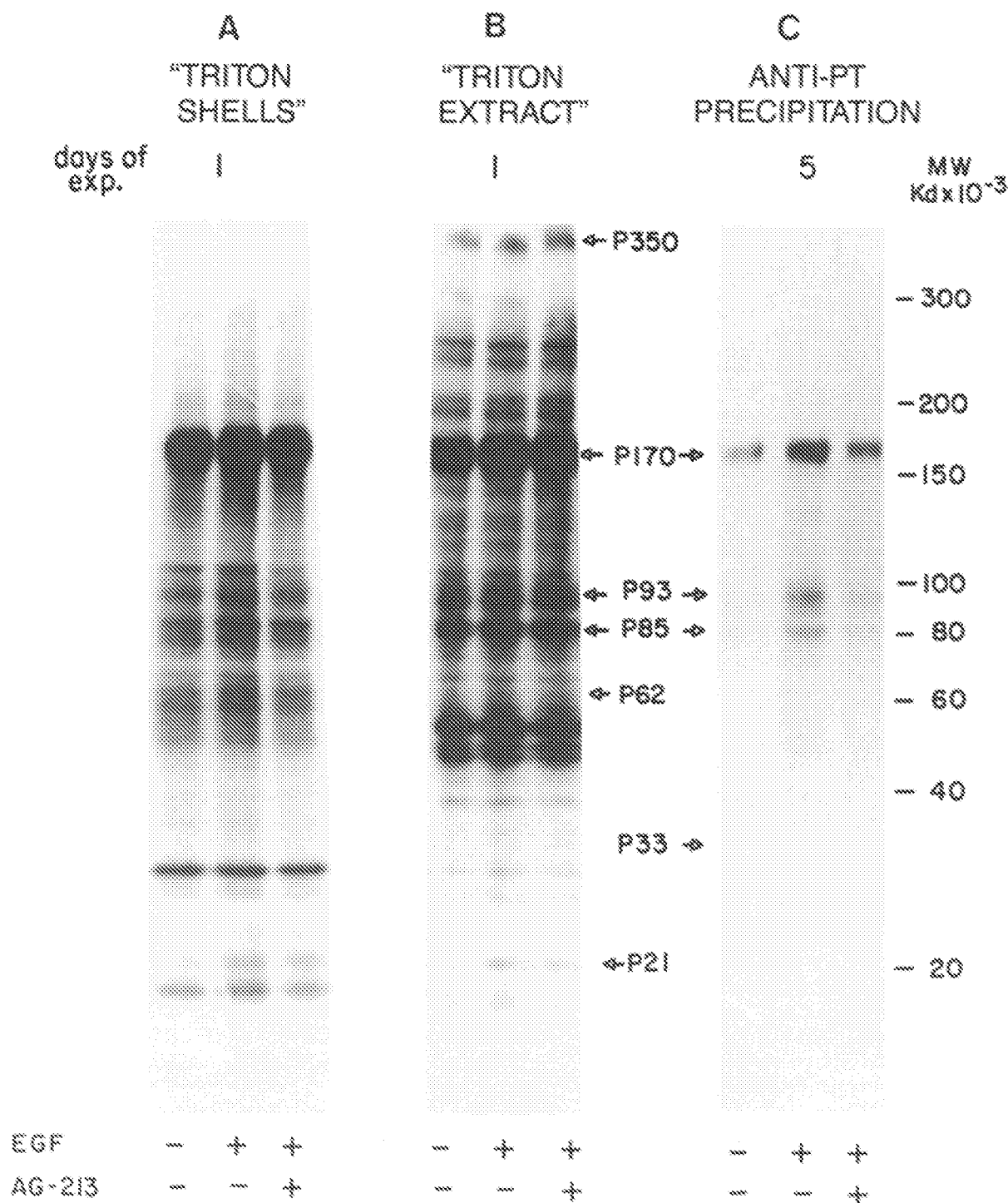


Figure 4. Effect of EGF and tyrphostins on total and tyrosine phosphate-containing polypeptide phosphorylation. Human keratinocyte cultures were grown as in Materials and Methods on 35-mm plates to 90% confluency. The cultures were treated as in Figs. 2 and 3 to induce in situ phosphorylation in the presence or absence of 10 ng/ml EGF or 100 μ M AG213 or both (see Materials and Methods). The resulting Triton X-100 extracts (cytosols and membrane proteins) were centrifuged 15 min at 10,000 g and either immunoprecipitated with antiphosphotyrosine antibodies or denatured in denaturation buffer for electrophoretic separation. The remaining "Triton shells" (cytoskeletons and nuclei) were scraped off the dishes with a rubber policeman and dissolved in denaturation buffer for SDS-PAGE on 5–15% gradient gels (27 cm long). Equal amounts in "cells equivalents" were loaded onto the gels for comparison. The gels were dried and autoradiographed for 1 or 5 d (see above). Note that tyrosyl phosphate-containing polypeptides were barely observed in the general phosphorylation profile (with the exception of P21 and P17). Major tyrosine phosphate-containing polypeptides are marked with open head arrows. These can be detected upon further exposure of the gels in C to X-ray film.

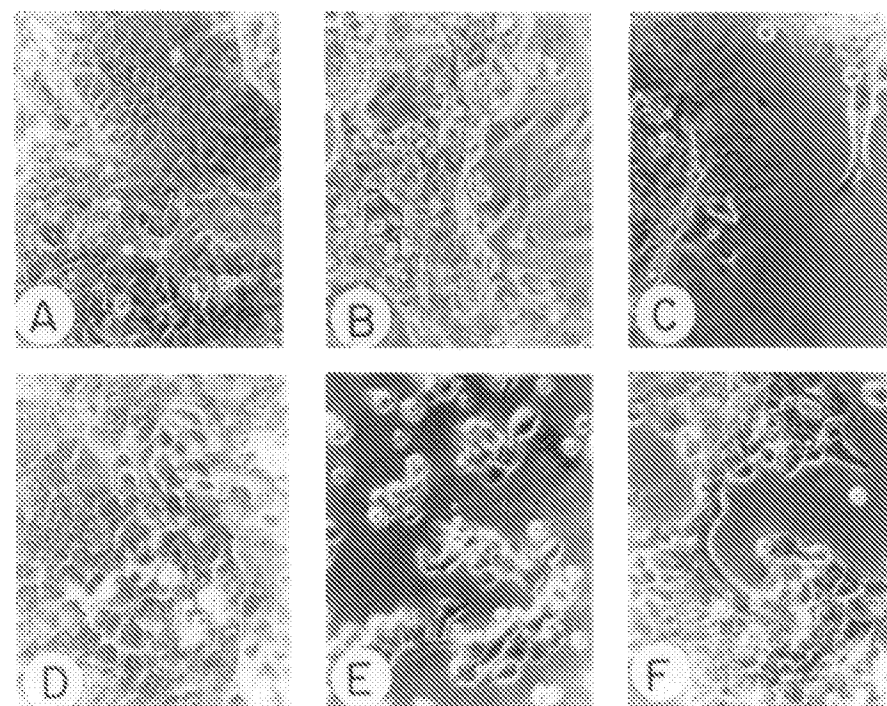


Figure 5. The reversible effect of tyrphostin AG213 (RG 50864) on human and guinea pig keratinocytes. Human and guinea pig keratinocyte cultures were grown as described in Materials and Methods, treated with tyrphostins, and photographed in a phase-contrast microscope (Olympus IMT-2 equipped with camera). In guinea pig cultures, incubation times were adjusted somewhat, relative to human-derived cultures. All pictures are shown at 200 \times . (A) Human cultures were control photographed 5 d after seeding cultures (^3H]thymidine incorporation in 12 h, 31.0 dpm/cell). (B) Human cultures with 50 μM AG213 added 2 d after seeding and photographed at 5 d. Large colony exhibiting enhanced terminal differentiation is shown. Cell rounding is more prominent in small colonies or single cells (see also Fig. 4 E). Fibroblasts of the 3T3 feeder layer seem unaffected (right) (^3H]thymidine incorporation, 16.2 dpm/cell). (C) Human cultures incubated with 50 μM AG213 as above for 24 h only, then washed and incubated for another 48 h with regular

medium; note the extensive outgrowth of proliferating keratinocytes at the edge of the colonies (^3H]thymidine incorporation, 27.6 dpm/cell). (D) Guinea pig keratinocytes control culture photographed after 72 h incubation (^3H]thymidine incorporation, 14.0 dpm/cell). (E) Guinea pig keratinocytes culture with AG213 (50 μM) added 4 h after seeding, photographed at 72 h (^3H]thymidine incorporation, 2.0 dpm/cell). (F) Guinea pig keratinocyte culture incubated with 50 μM AG213 as above for 24 h, then washed and incubated further 48 h with regular medium (^3H]thymidine incorporation, 12.0 dpm/cell).

gan culture in order to induce $\sim 50\%$ inhibition as compared to cell cultures (Fig. 1, Table I): 100 μM compared to 10 μM . (b) There is no tyrphostin inhibition of thymidine incorporation into dermal cells in comparison to the epidermal section of the skin and there is no EGF stimulation of ^3H]thymidine incorporation into dermal cells. (c) Tyrphostins action in arresting growth of epidermal keratinocytes is not

confined to normal human cells, but is also evident in guinea pig-derived epidermal keratinocytes, where similar results have been obtained (Figs. 5 F and 6 B). As mentioned above, in contrast to human keratinocytes that grow on irradiated NIH 3T3 feeder layer cells, guinea pig keratinocytes do not require the feeder layer for their growth in culture.

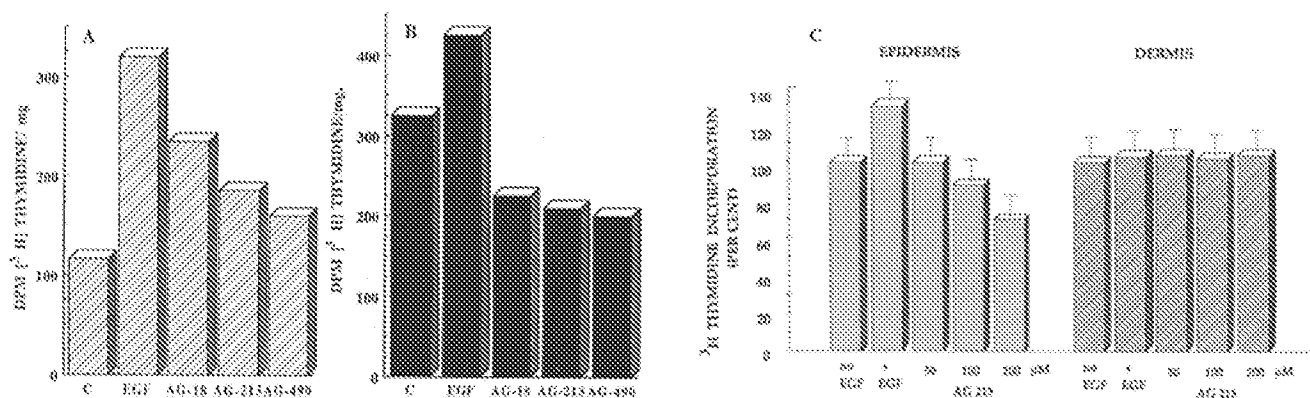


Figure 6. Inhibition of EGF-stimulated growth by tyrphostins in organ cultures. Organ Culture of human (A) and guinea pig (B) skin pieces was performed as described in Materials and Methods. Growth stimulation by EGF and its inhibition by three different tyrphostins is shown. (A) Human skin incubated 72 h with 100 μM of various tyrphostins in the presence of 10 ng EGF. One experiment of three is shown. (B) Guinea pig ear skin incubated with 10 nM EGF and 100 μM tyrphostins as in A. One experiment of three is shown. (C) Human skin incubated with 50, 100, and 200 μM AG213 for 48 h in the presence of 10 nM EGF, where the last 24 h were in the presence of ^3H]thymidine. The values are presented as an average of six independent experiments. 100% value (516–769 dpm/mg in the epidermis and 221–535 in the dermis) represents ^3H]thymidine incorporation in the absence of EGF. Stimulation by EGF varied between 12 and 43% above controls without EGF in various samples.

Discussion

Protein tyrosine phosphorylation comprises a very small portion of total phosphorylation of cellular proteins (14). Nevertheless, tyrosine phosphorylation plays a key role in signal transduction, resulting in cell growth. In numerous cases, hyperproliferation as well as cell transformation is associated with the enhanced activity of protein tyrosine kinases (5, 28, 29, 32). We have, therefore, reasoned that developing specific tyrosine kinase inhibitors could be of use in suppressing states of hyperproliferation and malignancy (17, 31).

Indeed, we could demonstrate that protein tyrosine kinase blockers from the tyrphostin family effectively inhibit the EGF-dependent proliferation of tissue culture cells that express EGF receptors. Furthermore, we found that their antiproliferative activity correlates well with their potency to inhibit EGF receptor kinase activity (12, 18, 31). In this study we show for the first time the inhibitory effect of tyrphostins on cultured normal keratinocytes (Figs. 1 and 5), and that the antiproliferative activities of the tyrphostins correlates very well with their potency to inhibit the EGF-dependent phosphorylation of the EGF receptor and of intracellular substrates (Figs. 2 and 3). This conclusion is further strengthened by the finding that the overall serine/threonine phosphorylation was not affected significantly by the tyrphostins used (Fig. 4) and that other important signal transducing systems, protein kinase C and cAMP-dependent protein kinase, were not inhibited by concentrations up to the millimolar range (data not shown). Furthermore, the inhibitory effects of these blockers is exerted directly on the keratinocytes without the mediation of the feeder layer cells (see Results).

From Fig. 6, which depicts the effect of the blockers on human and on guinea pig keratinocytes in organ culture, it is apparent that tyrphostins inhibit both EGF-dependent and EGF-independent growth. This phenomenon is also observed, though to a lesser extent, for human keratinocytes grown in cell culture. These seemingly puzzling findings can be accounted for by two alternative possibilities. (a) The inhibition by tyrphostins involves other type(s) of tyrosine kinase(s). (b) The so called EGF-independent portion of cell growth is mainly mediated via the action of auto-secreted TGF α (6, 7), and therefore also involves the EGF receptor (15).

One can argue that in organ culture the "mesenchymal effect" (13) of the dermal portion in the skin may induce EGF-independent growth of the keratinocytes. However, our ability to demonstrate similar tyrphostin inhibition in cultures without feeder layer and in guinea pig keratinocytes grown regularly without any feeder cells again shows that the keratinocyte alone can grow in an "EGF-independent" fashion, at least partially. Furthermore, EGF dependency also is not complete in keratinocyte cultures grown in different media too without feeder layers (19, 30, and this study).

The quantitative correlation between inhibition of EGF-dependent growth (Fig. 1), EGF-dependent receptor autophosphorylation (Fig. 2), and EGF-dependent tyrosine phosphorylation of intracellular substrates (Fig. 3) clearly implicates the EGF receptor in mediating the inhibitory effects of the above blockers, and strongly favors the second possibility. In permeabilized keratinocytes 80–95% of the apparent tyro-

sine phosphorylation observed is EGF dependent (Figs. 3 and 4), yet the tyrphostin's IC₅₀ values for inhibition of phosphorylation are identical to their IC₅₀ values observed for inhibition of cell growth and for the inhibition EGF receptor autophosphorylation (Fig. 2).

It seems, therefore, that the basal activity of the receptor might reflect autocrine activation by TGF α , which was reported to be produced in these cells (6, 7) and therefore most of EGF-independent growth signal seems to reflect TGF α -stimulated EGF receptor. Should this be the case, it could be postulated that the reduction of the EGF-independent signals as obtained in the "in situ" phosphorylation assays, stems from the washing of secreted TGF α present in the culture medium before the experiment. This possibility is also supported by the observation that anti-EGF antibodies (capable of cross-reactivity with TGF α molecules) inhibited some 30% of the EGF-independent portion of cell growth (data not shown). Furthermore, preliminary studies using ³²P-labeled cells followed by EGF-R immunoprecipitation showed high basal phosphorylation of EGF-R in the absence of added EGF in unstarved keratinocyte cultures (our unpublished data), thus, supporting the assertion that some EGF-R molecules are "autoactivated" by the putative autosecreted TGF α .

The arrest of keratinocyte growth by the PTK blocker tyrphostin AG213 (RG50864) is reversible and affects the proliferating keratinocytes (Fig. 5). This reversible action of tyrphostins AG213, and also of AG18 and AG490 (data not shown), and their nontoxicity (see Results) was also observed in EGF-dependent growth of other cultured cells harboring the EGF receptor and whose growth is strongly stimulated by EGF (11, 12, 18, 31).

From Fig. 1 it is apparent that the inhibitory IC₅₀ values for the three tyrphostins examined are in the range of 7–25 μ M, whereas the Ki values of these compounds are an order of magnitude lower (11, 12, 31). The higher concentrations required for the inhibition of EGF-dependent cell growth as compared to the activity of these compounds in vitro most probably reflects the presence of high affinity intracellular substrates which compete effectively with the tyrphostin. We have already observed previously that AG213 inhibits the EGF-dependent phosphorylation of PLC γ at rather high concentrations, probably because of the protein's tight association with the EGF receptor (20, 21).

We are currently attempting to design more potent tyrphostins and hydrophobic derivatives of these blockers in order to obtain compounds that are more active in intact cells. We have recently been able to demonstrate that a hydrophobic PDGF receptor kinase blocker AG17 (RG 50872) is a much more effective blocker of PDGF-dependent tyrosine kinase in intact rabbit vascular smooth muscle cells than a PDGF receptor kinase blocker in cell-free systems. Furthermore, the PDGF receptor kinase blocking activity in situ correlates quantitatively with its ability to inhibit PDGF-induced cell proliferation (4). In contrast, nonhydrophobic tyrphostins exhibit similar or higher inhibitory IC₅₀ values as blockers of PDGF-stimulated cell growth as compared with their inhibitory activity in vitro (4). We also observed previously that hydrophobic insulin receptor kinase blockers are more effective in intact cells than in vitro (25; Zik, Y., and A. Levitzki, unpublished results).

Our findings that tyrphostins arrest the growth of human keratinocytes, but do not possess large and significant toxic

adverse effects on the cells up to 200 μ M or higher (Figs. 4 and 5), make them strong candidates as agents against hyperproliferation of keratinocytes, which occurs in skin disorders such as psoriasis.

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